AD_____

COOPERATIVE AGREEMENT NUMBER DAMD17-96-2-6017

TITLE: A Novel Gene Gun-Mediated IL-12 Gene Therapy for Breast Cancer

PRINCIPAL INVESTIGATOR: Ning-Sun Yang, Ph.D.

CONTRACTING ORGANIZATION: PowderJect Vaccines, Inc.
Madison, Wisconsin 53711

REPORT DATE: October 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | October 1997 | Annual (15 Sep 96 - 14 Sep 97) |

**4. TITLE AND SUBTITLE**
A Novel Gene Gun-Mediated IL-12 Gene Therapy for Breast Cancer

**5. FUNDING NUMBERS**
DAMD17-96-2-6017

**6. AUTHOR(S)**
Ning-Sun Yang, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Powderject Vaccines, Inc.
Madison, Wisconsin 53711

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

**10. SPONSORING/MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200*

Advanced breast cancer is highly metastatic, and no methods exist to prevent relapse. Animal studies suggested that immunotherapy might achieve regression of both primary tumors and metastases and induce immunological memory to prevent recurrence. Unfortunately, many patients experienced toxicity from high systemic cytokine doses. In hopes of reducing toxicity, we and others tested localized cytokine administration via gene transfer. Using a gene gun to deliver interleukin 12 (IL-12) cDNA to the skin over a tumor site, we achieved partial to complete regression and antimetastatic effects in six non-breast mouse tumor models, without apparent toxicity.

We are now testing these protocols in two mouse models of mammary adenocarcinoma. In the moderately immunogenic TS/A model, we achieved complete regression in 50% of mice. Those mice remained tumor-free for an extended period, resisted secondary tumor challenge, and displayed augmented cell-mediated cytotoxicity, thus demonstrating immunological memory. In contrast, for the non-immunogenic 4T1 tumor, primary tumor growth was not affected by IL-12 gene therapy, although lung metastasis was significantly reduced. The anti-metastatic effect in the 4T1 model appears to be T cell-independent, and we are investigating its mechanism. These results suggest that a similar gene therapy protocol may be useful in human breast cancer treatment.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Breast Cancer | 32 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

NSY Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

NSY In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NSY In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

NSY In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature 10/10/97 Date

# TABLE OF CONTENTS

# A. INTRODUCTION

Invasive breast cancer is the leading form of cancer among women and the second biggest killer, after lung cancer (1). For a woman living in North America, the lifetime odds of getting breast cancer now stand at 1 in 8, double the risk of 1940 (1). Approximately 180,000 women were expected to be diagnosed with breast cancer in 1995 (2), with more than 40,000 expected to die from metastatic breast cancer (3).

Although substantial progress has been made in the detection and treatment of localized (nonmetastatic) disease, there has been relatively modest progress in the treatment of advanced disease. Thus, there is an urgent need for new, effective therapeutic approaches for metastatic breast cancer.

Cytokine therapy is regarded by many clinical investigators as one of the more promising approaches for treatment of advanced forms of cancers, including breast cancer, because it can be directed at eradication of both the primary tumor and its metastases via activation of the antitumor immunity. Among various cytokines, IL-12 in particular exerted dramatic antitumor effects in several different experimental tumor models (4,5). Unfortunately, little information is available for mammary tumor models. In addition, recombinant IL-12 protein in therapeutic doses can be toxic to mice and humans (6,7).

The overall goal of our research is to develop an immunological approach for breast cancer gene therapy that results in regression of both primary tumors and residual metastatic foci, and can induce sufficient immunological memory to prevent tumor recurrence and progression. Based upon our previous gene gun studies, this strategy is expected to exploit the gene therapy potential for treatment of breast cancer without the toxic side effects encountered in other studies employing cytokine protein therapy (6,7).

We recently reported (8,9) that gene gun-mediated *in vivo* delivery of IL-12 DNA elicited effective antitumor responses with no evident toxicity. This therapeutic effect was achieved via localized transgenic production of IL-12 protein, at a systemic level at least 1,000 times less than the effective, and toxic, dose of recombinant IL-12 protein delivered systemically (6,7,10). We therefore anticipated that gene gun-mediated IL-12 gene therapy might be effective and non-toxic in murine models of mammary cancer.

To our knowledge, there have been no reports on the use of *in vivo* gene therapy against murine mammary tumors. However, our previous study showed that six out of six tumor models tested, including two sarcomas, a renal cell carcinoma, a lymphoma, a melanoma and a mastocytoma, responded at varying degrees to gene gun-mediated IL-12 gene therapy *in vivo* (8). Responses varied from complete regression to a slowing of tumor growth, depending on the immunogenicity of each tumor. These syngeneic mouse tumor models resemble several currently employed murine mammary tumor models, for which non-immunogenic, poorly immunogenic, and highly immunogenic tumor cell lines have been characterized and histology, tumorigenicity, and metastatic capacity recently established (11-17). Thus there was reason to suggest that murine mammary tumors also may be responsive to this gene therapy protocol.

The purpose or the work reported here was to determine whether our previous success with *in vivo* IL-12 gene therapy could be translated to murine mammary cancer. We proposed to do this using three different murine mammary cancer models, reported to have different characteristics regarding immunogenicity, tumorigenicity, and metastatic potential. As described in the body of this report, we were able to carry out the planned experiments with two of the three murine mammary tumor lines (the third being contaminated with a virus that was not admissible in our mouse colony). The results to date have been encouraging, as described below.

# B. BODY OF REPORT

## B1. Experimental Methods, Assumptions, and Procedures

**Mice:** Balb/c mice were obtained from Harlan-Sprague Dawley (Madison, WI), and Balb/c nude mice from Taconic (Germantown, NY). Female mice 8-12 weeks of age were used in the experiments. Housing, care and use of mice were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, National Institutes of Health, Bethesda, MD, 1985).

**Mammary tumor models:** TS/A adenocarcinoma (11,17) and 4T1 adenocarcinoma (13) were kindly provided by G. Forni (Immunogenetic and Histocompatibility Center, Turin, Italy) and F.R. Miller (Michigan Cancer Foundation, Detroit, MI), respectively. Both cell lines were established from spontaneous, moderately differentiated mammary adenocarcinomas growing in Balb/c mice and are metastatic. Tumor cell cultures were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and gentamycin at 50 μg/ml. Before being injected into mice, tumor cells were detached from the plastic by a short incubation in Trypsin-EDTA solution. Mice were shaved in the abdominal area and injected intradermally (i.d.) with $1x10^5$ viable tumor cells in 50 μl Dulbecco's Phosphate Buffered Saline. For most *in vivo* experiments, 8 mice per group were tested; for survival experiments, 12-16 mice per group were evaluated. Growth of primary tumor was monitored 2-3 times a week by measuring two perpendicular tumor diameters using calipers. Tumor metastasis was estimated in three ways. Initially the metastatic tumor load of 4T1 cells was assessed by weighing the excised lungs on day 31 after tumor cell implantation, when it was noted that control mice had become moribund. In later experiments the primary tumor was surgically excised on day 11 post tumor cell implantation, and metastasis was estimated either directly by counting tumor foci in lungs stained with India ink, or indirectly by noting the survival time of mice in the various treatment (or no-treatment) groups.

**Transgene expression vectors:** We used plasmid pWRG3169, which contains the coding sequences for the p35 and p40 subunits of murine IL-12 arranged in the same orientation in tandem, with each driven by its own CMV promoter. This construction gave the highest level of IL-12 functional activity (stimulation of ConA-activated spleen cells) and immunoreactivity (by ELISA) when compared with other arrangements including a bi-cistronic construct with an internal ribosome entry site. This and a luciferase (Luc) cDNA expression vector have been described by us previously (8).

***In vivo* IL-12 gene transfer via gene gun:** For all *in vivo* skin transfection experiments, we utilized a helium-pulse *Accell*® gene gun (Powderject Vaccines, Inc., Madison, WI) as previously described (8). Briefly, plasmid DNA was precipitated onto gold particles (2.1 μm in diameter) and coated onto the inner surface of a Tefzel tubing (1/8" outside diameter, 0.93" inside diameter; McMaster-Carr, Chicago). The tubing was cut into half-inch segments, each segment being a gene delivery "cartridge". Conditions were adjusted so that each cartridge contained 0.5 mg of gold and 1.25 μg of plasmid DNA. For *in vivo* gene delivery, mouse skin

overlying and surrounding the target tumor was transfected with either the IL-12 cDNA or the Luc cDNA expression vector. Each treatment consisted of four transfection shots (i.e., 4 cartridges were used per treatment), containing a total of 5 μg of plasmid DNA. The DNA-coated gold particles were propelled by a helium gas pulse, at a discharge pressure of 300 pounds per square inch (psi). One transfection shot was delivered directly over the tumor site, and three additional transfection shots were placed at adjacent sites around the tumor site. This gene therapy procedure was performed 2-3 times per week for two weeks.

***In vitro*** **cellular immunology studies:** Experimental mice were treated with IL-12 cDNA or Luc cDNA on days 7 and 10 post 4T1 tumor cell implantation, and tumor draining (axillary) lymph nodes (TDLN) were taken on day 11. Lymph node cells ($2x10^6$) were incubated in culture medium without any stimuli at 37°C for 48 hr. Cell culture supernatants were collected and frozen at -20°C until testing. IFNγ levels in cell culture supernatants were measured by an ELISA kit (Endogen, Cambridge, MA). For the tumor cell proliferation assay, $2x10^4$ 4T1 tumor cells in triplicates were incubated for 48 hr in the absence or presence of serial dilutions of TDLN cell-derived culture supernatants. In some wells, recombinant mouse IFNγ (Endogen, Cambridge, MA) and/or anti-mouse IFNγ mAb ( Pharmingen, San Diego, CA) were added. The tumor cells were labeled with $^3$H-thymidine for 6 hr, trypsinized and harvested. Radioactivity was determined using a Packard liquid scintillation counter. Proliferation Inhibition Index was calculated according to the formula:

$$\frac{\text{Proliferation in medium without sample - Proliferation in medium with sample}}{\text{Proliferation in medium without sample}} \times 100\%.$$

**Assumptions:**

- We assume that the two murine adenocarcinoma tumor-cell lines used in these studies are each representative of part of the range of human breast cancers, with regard to immunogenicity and metastatic potential. This assumption is discussed further in the Discussion section, Part B3.
- We started with the assumption that the protocols we developed previously for other histological murine tumor types (two sarcomas, a renal cell carcinoma, a lymphoma, a melanoma and a mastocytoma) would likely be applicable to the transplantable mammary tumor lines used in this study. This assumption seems to have been borne out, as shown in Results, Part B2.
- We did <u>not</u> assume that previously reported information regarding the degree of immunogenicity of the TS/A and 4T1 murine mammary tumor lines would apply in our hands and under our experimental conditions. Before beginning the planned IL-12 gene therapy experiments, we performed experiments to test the immunogenicity of these two tumor lines.

## B2. Results

### Immunogenicity of TS/A and 4T1 tumors.

Since human breast cancer, like most of the other types of cancer, is considered to be poorly immunogenic (18), we decided to employ for our study the weakly immunogenic or non-immunogenic murine mammary tumors. TS/A adenocarcinoma was described as non-immunogenic in some studies (11) and as poorly immunogenic in later studies by the same authors (19). We could not find a published reference on the immunogenicity of the 4T1 tumor, although it was personally communicated as non-immunogenic by Dr. F. Miller (Michigan Cancer Foundation, Detroit, Michigan). Therefore, we performed preliminary experiments to test the immunogenicity of these two adenocarcinomas. Balb/c mice were immunized i.d. twice at a 3-week interval with $10^6$ gamma-irradiated (5,000 rad) TS/A or 4T1 adenocarcinoma cells. One month after the last immunization, mice were challenged i.d. with $10^5$ viable tumor cells, and tumor growth was followed. Fig. 1 shows that whereas TS/A tumors did not grow in the mice pre-immunized with irradiated TS/A cells, 4T1 tumors grew in pre-immunized mice at the same rate as in control mice. Our data confirm, therefore, that TS/A is an immunogenic tumor, and 4T1 is apparently non-immunogenic.

### Effects of IL-12 gene therapy on the growth of primary TS/A and 4T1 tumors.

We next evaluated the antitumor effect of gene gun-mediated IL-12 gene therapy on established, intradermal TS/A and 4T1 tumors. The mice were treated according to a standard protocol that we previously established (8), starting on day 7 after tumor cell implantation. IL-12 gene therapy resulted in a substantial antitumor effect against the TS/A tumor (Fig. 2A), in that 50% of treated mice rejected established tumors (~4 mm average diameter at the start of treatment). This experiment was repeated twice more, with similar results. Also, similar to what we reported in a previous study with other immunogenic tumors (8), mice that rejected TS/A tumors remained tumor-free for 2 months resisted a second challenge with the same tumor cells (Fig. 3) and were able to generate an augmented CTL response *in vitro* (Fig. 4). In contrast to the TS/A tumors, the growth of the 4T1 primary tumors (Fig. 2B) was not affected by IL-12 gene therapy, consistent with the apparent non-immunogenic nature of this tumor.

### Localized IL-12 gene delivery resulted in a systemic anti-metastatic effect against 4T1 tumor.

The 4T1 tumor line has been characterized as highly metastatic to the lungs and lymph nodes (13 ). During our experiments with therapy of intradermal primary tumors, as described above, we noticed that, on day 31 after tumor cell implantation, the Luc cDNA-treated (control) mice looked moribund, whereas the IL-12 cDNA-treated mice did not have visible signs of distress. When the mice were sacrificed and their lungs inspected, we noticed a striking difference in the size of the lungs and in the spread of metastatic nodules (Fig. 5). The anti-metastatic effect of IL-12 gene therapy was further analyzed by weighing the lungs. Table 1 shows that the average lung weight in IL-12-treated mice was significantly lower than that of the Luc-treated mice or the

untreated tumor-bearing mice. The observed effect against spontaneous metastases was further confirmed by two additional criteria: (a) the number of metastatic nodules in the lungs and (b) the survival time of mice following primary tumor excision.

To compare the number of tumor nodules in the lungs, mouse skin was transfected with either IL-12 cDNA or Luc cDNA on days 7,10,14,17 and 20 after 4T1 tumor cell implantation. Mice were sacrificed on day 21 and immediately injected intratracheally with India Ink followed by lung fixation in Feketr's solution as described (20). Mice treated with the Luc cDNA (n=8) were scored to contain $24.5 \pm 3.8$ visible tumor nodules in their lungs, whereas mice treated with the IL-12 cDNA (n=5) had $11.8 \pm 1.3$ tumor nodules ($p<0.01$).

For animal survival studies, we first performed an experiment to determine the time course of spontaneous metastasis of 4T1 tumor to the lungs. The primary tumors were surgically removed on day 7, 11 or 17 post tumor cell implantation (n=8 in each group), and survival time of the mice was followed. When the tumor was removed on day 7, 62.5% of mice survived for at least 120 days, whereas all mice with tumors removed on day 11 or 17 died by day 41 after tumor cell implantation. These results show that 4T1 tumors start to metastasize to the lungs during the first week of tumor growth, and that by day 11 virtually all mice have metastases that are destined to be lethal. Therefore, in the following experiments we excised the primary tumor on day 11 post tumor cell implantation. The results of two independent experiments on the time course of mouse survival are presented in Fig. 6. Mice were untreated or transfected in the skin overlying an intradermal tumor with the IL-12 cDNA or Luc cDNA expression vector on day 3 (Exp.1) or day 4 (Exp.2), and then on days 7 and 10 after 4T1 tumor cell implantation, followed by tumor excision. The combined data from these two experiments show that 5 of 28 mice (17.9%) treated with IL-12 cDNA survived the spontaneous metastases and were considered "cured", whereas only 1 of 28 (3.6%) in each of the control groups survived until day 120 (the final observation day of that experiment). This difference between the IL-12 group and the pooled control groups, 5/28 versus 2/56 surviving, was statistically significant by the Fisher Exact Test ($P = 0.038$). Among the mice that died during the course of the experiments, the IL-12-treated mice lived significantly longer than the Luc-treated or untreated mice ($48.48 \pm 2.43$, $40.92 \pm 1.52$ and $37.14 \pm 0.72$ days, respectively; $p<0.025$). Mice that survived metastasis were, however, not able to resist a challenge with $10^5$ 4T1 tumor cells (data not shown). Thus protective immunological memory was not induced.

**Anti-metastatic effect of IL-12 gene therapy in nude mice.**

We have previously reported (8) that the regression of immunogenic tumors induced by the current gene gun-mediated IL-12 gene therapy protocol is CD8+ T cell-dependent. However, the apparent inability of 4T1 tumor to induce a specific immune memory response as described above argues against the observed anti-metastatic effect of IL-12 gene therapy being due to a classical T-cell response. To directly assess the role of T cells, we injected 4T1 cells i.d. into athymic nude mice in a parallel experiment with the normal euthymic Balb/c mice, and treated the mice with our standard IL-12 gene therapy protocol followed by evaluation of lung metastases. Fig. 7 shows that IL-12 gene therapy resulted in a similar anti-metastatic effect in nude mice as can be obtained in euthymic mice, suggesting that T cells were likely not involved

in the anti-metastatic effect observed in the 4T1 tumor model. This conclusion is supported by a T-cell depletion experiment in which 4T1 tumor-bearing mice were treated with either: (a) Luc DNA, (b) IL-12 DNA plus control immunoglobulin, or (c) IL-12 DNA plus antibodies to murine CD4+ and CD8+ T cells (Fig. 8).

**Activation of tumor-draining lymph node cells following IL-12 gene therapy**.

Since it appears that T cells may not play a role in the observed systemic anti-metastatic effect of IL-12 gene therapy, and because IL-12 protein did not exhibit any inhibitory effect on the growth of 4T1 tumor *in vitro* (data not shown) or *in vivo* (Fig. 1), our working hypothesis is that transgenic IL-12 may have acted by stimulating or augmenting some local or regional nonspecific host defense mechanisms that could have actively suppressed 4T1 tumor metastasis. It has been shown previously that following subcutaneous implantation, 4T1 tumor first metastasizes into the regional lymph nodes and than into the lungs (13). We hypothesized, therefore, that IL-12 gene therapy may induce an immune or inflammatory reactivity in tumor-draining lymph nodes (TDLN) which in turn could inhibit tumor growth, invasion, or spreading.

To test this possibility, we first evaluated IFNγ production in TDLN, because it has been previously shown that IL-12 can readily stimulate IFNγ production (5,21,22), and IFNγ can be directly cytotoxic to tumor cells (23). Mice were injected i.d. in the middle of abdominal area with $10^5$ 4T1 tumor cells, and skin-transfected with IL-12 cDNA or Luc cDNA 7 and 10 days later. On day 11, the draining (axillary) lymph nodes were removed, and the derived cells ($2x10^6$) were incubated *in vitro* without any stimulation at 37°C for 48 hr. The cell-free supernatants (conditioned media) were assayed for IFNγ by ELISA. Table 2 shows that IL-12 gene therapy resulted in a substantially enhanced production of IFNγ in TDLN as compared with mice treated with Luc cDNA, or untreated tumor-bearing mice.

We then determined whether the conditioned cell culture supernatants of TDLN possessed inhibitory activity for tumor cell proliferation following gene therapy. The cell-free supernatants at various serial dilutions were added to 4T1 tumor cells in culture, and the effect on proliferation of these cells was determined. Fig. 9A shows that culture supernatants from TDLN of IL-12-treated mice inhibited the proliferation of 4T1 cells in a dose-dependent manner. In contrast, culture supernatants of TDLN cells from Luc-treated mice did not show a significant effect on tumor cell proliferation. The observed tumor inhibitory activity was not neutralized by the addition of anti-IFNγ mAb to the cell supernatants, whereas the same mAb neutralized the tumor inhibitory activity of the recombinant murine IFNγ (rMuIFNγ) protein (Fig. 9B). In this comparative experiment, rMuIFNγ was used at a concentration of 6 ng/ml, which was similar to the IFNγ concentration in the pooled TDLN cell supernatant at 1:4 dilution (Fig. 9A,B), namely 8-10 ng/ml, based on two separate determinations.

## B3. Discussion

The leading cause of death of women with breast cancer is tumor metastases in visceral organs (1-3). In this study we show that *in vivo* gene therapy with IL-12 can result in complete regression of in 50% of cases of the immunogenic, metastatic murine TS/A adenocarcinoma growing intradermally. In contrast, using the same gene therapy procedure, we found no effect of IL-12 against the apparently non-immunogenic, intradermal 4T1 tumor. Remarkably, however, a significant reduction of lung metastases was observed in 4T1 tumor-bearing mice as a result of IL-12 gene therapy. Therefore, the data obtained in this study suggest that the current gene gun-mediated IL-12 gene therapy protocol may have clinical application to future treatment of human breast cancers.

The understanding of tumor immunogenicity is crucially important for developing rational design of cancer immunotherapy in humans. The murine tumors used in this study, TS/A and 4T1 mammary adenocarcinomas, were tested for immunogenicity; we found the TS/A tumor to be immunogenic, and 4T1 to be non-immunogenic under the conditions used here. In two previous studies, TS/A was initially described as non-immunogenic (11,24), but later as poorly immunogenic (19), depending on the assays used to test immunogenicity. It is apparent, therefore, that the definition of tumor immunogenicity in cancer immunotherapeutic studies needs to be standardized. In the classical animal studies, a tumor is considered to be immunogenic if it induces, following either surgical excision or vaccination, protection against a secondary tumor challenge *in vivo* (25). However, tumors originally classified as "non-immunogenic" by this criterion, after being biologically or transgenically modified, were often able to induce an immune response even against the unmodified tumor cells (26-29). Based on such studies, it is now believed that many tumors express tumor-associated antigens (TAA) which can be recognized by the immune system. Therefore, a tumor which is considered to be non-immunogenic based on immunization-challenge experiments, such as the 4T1 adenocarcinoma in our study, may be later characterized as poorly immunogenic if it could be modified to induce an immune response. We are currently investigating a strategy that is designed to generate a T cell-mediated immune response against the 4T1 tumor, in hopes of generating an even stronger anti-metastatic effect when combined with the current IL-12 gene therapy protocol.

It has long been claimed that most human tumors are non-immunogenic because of their spontaneous origin, and, therefore, that immunogenic murine tumor cell lines were invalid as models to provide therapeutic applications for human cancers (30). However, recent progress in tumor and cellular immunology has provided good evidence that some, if not all, human tumors are immunogenic, as defined by their expression of TAA that can be recognized by T lymphocytes (28,29,31). For human breast tumors, the best characterized TAA are HER-2/neu (32), p53 (33) and DF3/MUC-1 (34), and these antigens have been shown to be capable of inducing cytotoxic T cell responses in patients (35).

As a result, immunotherapeutic strategies for treatment of metastatic breast cancers have received increased attention in recent years. One such approach involves the use of recombinant

cytokines with the purpose of boosting the existing antitumor immune response. Studies in animal models have shown that IL-12, when compared with other cytokines, has outstanding antitumor efficacy (4,5,9), as was expected based on its known immune stimulatory effects on Th1 cells, cytotoxic T-cells and natural killer cells (36). Based on these studies, clinical trials with IL-12 were initiated. Unfortunately, the first clinical trial protocol revealed substantial toxicity of recombinant IL-12 protein in humans, resulting in the death of two patients and severe side effects in 15 others (7). Although the lack of a pre-dosing schedule was suggested as the cause for this toxicity, recent experiments have also suggested a need to explore alternative IL-12 delivery mechanisms, to assure treatment that is both safe and effective (37). Using an IL-12 gene therapy approach, we have shown that localized *in vivo* IL-12 gene transfer into skin tissue can result in eradication of established murine tumors and their metastases, leading to the generation of a strong tumor-specific immunological memory (8). More importantly, no signs of IL-12 toxicity were observed following this gene gun-mediated therapy protocol (9).

In the current study, we are extending our research on cancer gene therapy to breast cancer model systems. Results summarized above show that two murine mammary tumors are susceptible to IL-12 gene therapy. Complete tumor regression was achieved in the TS/A tumor model and was accompanied by generation of immunological memory, similar to our findings with other immunogenic tumors as reported previously (8). In contrast, the same gene therapy protocol did not alter the growth of the 4T1 tumor. These findings, together with the results of our previous study showing that even the growth of the weakly immunogenic B16 tumor was partially suppressed by IL-12 gene therapy (8), add further support to our confirmation that the 4T1 tumor is apparently non-immunogenic in nature.

Because 4T1 does appear to be non-immunogenic by multiple criteria, including the lack of effect of IL-12 gene therapy, we were surprised that the same therapy significantly reduced its the degree of metastasis to the lungs. These data are unprecedented, to our knowledge, and imply that the lack of a therapeutic effect on the primary solid tumor does not necessarily mean the absence of antitumor effect. In addition, we show in this study that, in contrast with the T cell-dependent antitumor effect of IL-12 gene therapy against immunogenic tumors (8), the anti-metastatic effect against the 4T1 tumor appeared to be not T cell-mediated. These results are in agreement with the studies using some other cytokine gene therapy strategies, which showed that the antitumor responses may not involve T cells (27).

The mechanisms of the anti-metastatic effect of IL-12 gene therapy in the 4T1 tumor model are not clear at this time. It appears that T cell activities including T cell-mediated cytotoxicity are not involved, since an anti-metastatic effect of similar magnitude was also observed in T cell deficient (both nude and T cell-depleted) mice. Knowing that 4T1 cells metastasize from a subcutaneous deposit first to TDLN and then into lungs and other organs (13), we hypothesized that some local or regional processes, induced by transgenic IL-12, may be responsible for the reduction of tumor metastasis into the lungs. Indeed, we found in a preliminary experiment that following IL-12 gene therapy, TDLN cells produced factor(s) that reduce the proliferation of 4T1 tumor cells *in vitro*. This augmented tumor inhibitory activity was not detected in the spleens of control DNA-treated or un-treated mice (data for un-treated mice not shown). These results suggest that the immune activation in TDLN induced by IL-12 gene therapy may restrict or

inhibit tumor metastasis from the primary tumor site into the lungs. The biochemical nature of the substances in TDLN accountable for the observed tumor-inhibitory effect has yet to be determined. We showed in this study, in agreement with others (38), that increased levels of IFNγ were produced in TDLN following IL-12 gene therapy. It is well known that IFNγ is induced by IL-12 (5,21,22) and can confer direct cytotoxicity to tumor cells (23). Indeed, recombinant murine IFNγ did inhibit the proliferation of 4T1 cells *in vitro* (Fig. 6B), whereas recombinant IL-12 did not (data not shown). However, a preliminary mAb neutralization experiment showed that the tumor-inhibitory factor released from TDLN cells in culture is apparently not IFNγ.

In Results, in discussing the lack of apparent IL-12 toxicity with our IL-12 gene therapy protocol, we briefly mentioned that, compared to IL-12 protein therapy, we are delivering much lower systemic levels (~1000-fold) in a localized manner at the tumor site. Another distinct feature of the gene therapy approach is that we are achieving a slow, continuous release of IL-12 over a period of several days. Thus it is not clear whether the observed anti-metastatic effect in the lungs is due solely to the presence of the transgenic IL-12 protein, or may also be due to the slow, continuous release achieved via the epidermal gene transfection. For example, it is possible that the slow release of localized, transgenic IL-12 protein can activate regional immunity in a different way than the bolus injection of rIL-12 protein which would rapidly diffuse under *in vivo* conditions. The mechanism of the observed phenomenon - an efficacious anti-metastatic activity in the absence of T cell immunity - hence warrants further investigation.

We suggest, based on this and previous studies (8,9), that gene gun-mediated *in vivo* IL-12 gene therapy approach may be developed as an effective and safe alternative to systemic IL-12 protein therapy. The TS/A and 4T1 mammary tumor cell lines used in this study, and the different antitumor effect of IL-12 gene therapy on primary tumors versus visceral metastases, may provide a highly desirable experimental model for immunotherapy and gene therapy studies related to human breast cancer. Our specific strategy for future research is to increase the anti-tumor immune response either by combining the IL-12 gene pair with other cytokine (IL-2, TNF) genes *in vivo*, or by increasing tumor cell immunogenicity via *ex vivo* transfection with IFNγ, GM-CSF, or B7-1 genes. We believe that extension of this study may provide an experimental rationale for proceeding to a clinical trial of gene gun-mediated IL-12 gene therapy for breast cancer.

## B4. Recommendations in Relation to the Statement of Work

The work completed to date strongly supports our expectation that IL-12 gene therapy protocols developed in murine cancer models of other histological types, and found to be effective in those tumor models, would have similar anti-tumor effects in murine models of mammary cancer. The most significant obstacle encountered to date is that one of the three proposed murine mammary cancer models, DA-DMBA-3, could not be used in these studies because of viral contamination. To safeguard the integrity of our mouse colony, we routinely test tumor lines for viral infections before using them in animal experiments. The DA-DMBA-3 line tested positive for parvovirus, and we have received no response to repeated requests for a second vial that may have been frozen before the viral infection occurred.

Another difference from the Statement of Work is that some experiments have been done sooner than originally projected. Specifically, we performed some preliminary experiments on anti-metastatic effect of IL-12 gene therapy against the 4T1 tumor and T cell involvement in this effect earlier than originally planned because the first experiment had shown very interesting and unexpected results and because we were curious to obtain a preliminary indication regarding the cellular mechanisms of the anti-metastatic effect against a non-immunogenic tumor.

Otherwise, we plan to follow the Statement of Work as originally outlined. Thus, in the coming year, we plan to:

- Determine whether a standard *ex vivo* gene gun transfection protocol developed in our laboratory can be usefully employed for transfection or co-transfection of non-immunogenic 4T1 tumor cells with B7-1, IL-12, IFNγ or other immune modulatory molecules, resulting in increased tumor immunogenicity and augmented antitumor effect of IL-12 gene therapy.
- Evaluate the role of NK cells in the observed effect of IL-12 gene therapy against the 4T1 tumor.

## C. CONCLUSIONS

- The TS/A mammary adenocarcinoma is moderately immunogenic, whereas the 4T1 mammary adenocarcinoma is apparently non-immunogenic.

- IL-12 gene therapy of the immunogenic TS/A adenocarcinoma results in complete regression of 50% of established primary tumors and induction of immunological memory.

- IL-12 gene therapy of the non-immunogenic 4T1 adenocarcinoma does not significantly affect the growth of the primary tumor, but reduces metastasis into the lungs.

- A brief course of IL-12 gene therapy significantly extends mouse survival time following excision of a 4T1 primary tumor.

- The anti-metastatic effect of IL-12 gene therapy against the 4T1 tumor appears not to be T cell-mediated.

## D. REFERENCES


1. Marshall, E. Search for a Killer: Focus Shifts From Fat to Hormones. Science 259, 618-621 (1993).

2. Shapiro, T. and Clark, P. Breast Cancer: What the Primary Care Provider Needs to Know. Nurse Pract. USA 20, 36, 39-40, 42 (1995).

3. Lippman, M. The Development of Biological Therapies for Breast Cancer. Science 259, 631-632 (1993).

4. Brunda, M., Luistro, L., Warrier, R., Wright, R., Hubbard, B., Murphy, M., Wolf, S. and Gately, M. Antitumor and Antimetastatic Activity of Interleukin 12 against Murine Tumors. J Exp. Med. 178, 1223-1230 (1993).

5. Nastala, C., Edington, H., McKinney, T., Tahara, H., Nalesnik, M., Brunda, M., Gately, M., Wolf, S., Schreiber, R., Storkus, W. and Lotze, M. Recombinant IL-12 Administration Induces Tumor Regression in Association with IFN-g Production. J Immunology 153, 1697-1706 (1994).

6. Orange, J., Salazar-Mather, T., Opal, S., Spencer, R., Miller, A., McEwen, B. and Biron, C. Mechanism of Interleukin 12-mediated Toxicities during Experimental Viral Infections: Role of Tumor Necrosis Factor and Glucocorticoids. J. Exp. Med. 181, 901-914 (1995).

7. Marshall, E. Cancer Trial of Interleukin-12 Halted. Science 268, 1555 (1995).

8. Rakhmilevich, A.L. *et al.* Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors. Proc. Natl. Acad. Sci. USA 93, 6291-6296 (1996).

9. Rakhmilevich, A.L., Janssen, K., Turner, J., Culp, J. & Yang, N.-S. Cytokine gene therapy of cancer using gene gun technology: Superior antitumor activity of IL-12. Human Gene Therapy 8, 1303-1311 (1997).

10. Noguchi, Y., Chen, Y.N. and Old, L. A Mouse Mutant p53 Product Recognized by CD4+ and CD8+ T cells. Proc. Natl. Acad. Sci. USA 91, 3171-3175 (1994).

11. Nanni, P., De Giovanni, C., Lollini, P.-L., Nicoletti, G. and Prodi, G. TS/A: a New Metastasizing Cell Line from a BALB/c Spontaneous Mammary Adenocarcinoma. Clin. Exp. Metast. 1, 373-380 (1983).

12. Cavallo, F., Di Pierro, F., Giovarelli, M., Gulino, A., Vacca, A., Stoppacciaro, A., Forni, M.,

Modesti, A. and Forni, G. Protective and Curative Potential of Vaccination with Interleukin-2 Gene-transfected Cells from a Spontaneous Mouse Mammary Adenocarcinoma. Cancer Res. 53, 5067-5070 (1993).

13. Aslakson, C. and Miller, F. Selective Events in the Metastatic Process Defined by Analysis of the Sequential Dissemination of Subpopulations of a Mouse Mammary Tumor. Cancer Res. 52, 1399-1405 (1992).

14. Sotomayor, E., Fu, Y.X., Lopez-Cepero, M., Herbert, L., Jimenez, J., Albarracin, C. and Lopez, D. Role of Tumor-Derived Cytokines on the Immune System of Mice Bearing a Mammary Adenocarcinoma. J. Immunology 147, 2816-2823 (1991).

15. Hossain, A., Sarkar, A. and Sarkar, N. Mixed Inocula of Mouse Mammary Tumour Cell Subpopulations Result in Changes of Organ-Specific Metatasis. Clin. Expl. Met. 9, 6, 501-515 (1991).

16. Lollini, P. L., Bosco, M., Cavallo, F., De Giovanni, C., Giovarelli, M., Landuzzi, L., Musiani, P., Modesti, A., Nicoletti, G., Palmieri, G., Santoni, A., Young, H., Forni, G. and Nanni, P. Inhibition of Tumor Growth and Enhancement of Metastasis after Transfection of the g-Interferon Gene. Int. J. Cancer 55, 320-329 (1993).

17. Pericle, F., Giovarelli, M., Colombo, M., Ferrari, G., Musiani, P., Modesti, A., Cavallo, F., Di Pierro, F., Novelli, F. and Forni, G. An Efficient Th2-Type Memory Follows CD8+ Lymphocyte-Driven and Eosinophil-Mediated Rejection of a Spontaneous Mouse Mammary Adenocarcinoma Engineered to Release IL-4. J. Immunology 153, 5659-5673 (1994).

18. Apostolopoulos, V., McKenzi, I.F.C., and Pietersz, A. Breast cancer immunotherapy: current status and future prospects. Immunology and Cell Biology 74, 457-464 (1996).

19. Alllione, A., Consalvo, M., Nanni, P., Lollini, P.L., Cavallo, F., Giovarelli, M., Forni, M., Gulino, A., Colombo, M.P., Dellabona, P., Hock, H., Blankenstein, T., Rosenthal, F.M., Gansbacher, B., Bosco, M.C., Musso, T., Gusella, L. and Forni, G. Immunizing and curative potential of replicating and nonreplicating murine mammary adenocarcinoma cells engineered with interleukin (IL)-2, IL-4, IL-6, IL-7, IL-10, tumor necrosis factor , granulocyte-macrophage colony stimulating factor, and -Interferon gene or admixed with conventional adjuvants. Cancer Research 54, 6022-6026 (1994).

20. Wexler, H. Accurate Identification of experimental pulmonary metastases. J. Natl. Cancer Inct., 36, 641-645 (1966).

21. Gately, M., Warrier, R., Honasoge, S., Carvajal, D., Faherty, D., Connaughton, S., Anderson, T., Sarmiento, U., Hubbard, B. and Murphy, M. Administration of Recombinant IL-12 to Normal Mice Enhances Cytolytic Lymphocyte Activity and Induces Production of IFN-γ in vivo. International Immunology 6, 157-167 (1994).

22. Sykes, M., Szot, G.L., Nguyen, P.L. & Pearson, D.A. Interleukin-12 inhibits murine graft-versus-host disease. Blood 6, 2429-2438 (1995).

23. Brunda, M.J. *et al.* Role of interferon-g in mediating the antitumor efficacy of interleukin-12. J. Immunother. 17, 71-77 (1995).

24. Bosco, M., Giovarelli, M., Forni, M., Modesti, A., Scarpa, S., Masuelli, L., and Forni, G. Low doses of IL1-4 injected perilympahtically in tumor-bearing mice inhibit the growth of poorly and apparently nonimmunogenic tumors and induce a tumor-specific immune memory. The Journal of Immunology 145, 3136-3143 (1990).

25. Middle, J.G. and Embleton, M.J. Naturally arising tumors of the inbred WAB/Not rat strain. JNCI 67 (3), 637-643 (1981).

26. Gansbacher, B., Zier, K., Daniels, B., Cronin, K., Bannerji, R. and Gilboa, E. Interleukin 2 Gene Transfer into Tumor Cells Abrogates Tumorigenicity and Induces Protective Immunity. J Exp. Med. 172, 1217-1224 (1990).

27. Golumbek, P., Lazenby, A., Levitsky, H., et al. Treatment of Established Renal Cancer by Tumor Cells Engineered to Secrete Interleukin-4. Science 254, 713-716 (1991).

28. Pardoll, D. New strategies for active immunotherapy with genetically engineered tumor cells. Current Opinion in Immunol. 4, 619-623 (1992).

29. Colombo, M.P., Modesti, A., Parmiani, G. and Forni, G. Local cytokine availability elicits tumor rejection and systemic immunity through granulocyte-T-lymphocyte cross-talk. Cancer Res. 52, 4853-4857 (1992).

30. Scott, O.C.A. Tumor transplantation and tumor immunity: a personal view. Cancer Research 51, 757-763 (1991).

31. Colombo, M. and Forni, G. Cytokine Gene Transfer in Tumor Inhibition and Tumor Therapy: Where are we now? Immunology Today 15, 48-51 (1994).

32. Disis, M.L., Calenoff, E., McLaughlin, G., Murphy, A.E., Chen, W., Groner, B., Jeschke, M., Lydon, N., McGlynn, E., and Livingston, R.B. et al. Existent T-cell and antibody immuntiy to HER-2/neu protein in patients with breast cancer. Cancer Research 54(1), 16-20 (1994).

33. Tilkin, A.F., Lubin, R., Soussi, T., Lazar, V., Janin, N., Mathieu, M.C., Lefrere, I., Carlu, C., Roy, M., and Kayibanda et al. Primary proliferative T cell response to wild-type p53 protein in patients with breast cancer. European Journal of Immunology 25(6), 1765-1769 (1995).

34. Gimmi, C.D., Morrison, B.W., Mainprice, B.A., Gribben, J.G., Boussiotis, V.A., Freeman, G.J., Park, S.Y., Watanabe, M., Gong, J., Hayes, D.F., Kufe, D.W., and Nadler, L.M. Breast

cancer-associated antigen, DF3/MUCi, induces apoptosis of activated human T cells. Nature Medicine 2(12), 1367-70 (1996).

35. Jerome, K.R., Domenech, N. and Finn, O.J. Tumor-specific cytotoxic T cell clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. The Journal of Immunology 151, 1654-1662 (1993).

36. Brunda, M.J. & Gately, M.K. Interleukin-12: Potential role in cancer therapy. *Important Adv. Oncology* , 3-18 (1995).

37. Coughlin, C.M., Wysocka, M., Trinchieri, G., and Lee, W.M.F. The effect of interleukin 12 desensitization on the antitumor efficacy of recombinant interleukin 12. Cancer Research 57, 2460-2467 (1997).

38. Bramson, J.L., Hitt, J., Addison, C.L., Muller, W.J., Gauldie, J., and Graham, F.L. Direct intratumoral injection of an adenovirus expressing interleukin-12 induces regression and long-lasting immunity that is associated with highly localized expression of interleukin-12. Human Gene Therapy 7, 1995-2002 (1996).

Table 1. Anti-metastatic effect of IL-12 gene therapy against a nonimmunogenic 4T1 tumor.

| Exp | Treatment[a] | No mice | Lung Weight (g)[b] Mean ± SEM | P |
|---|---|---|---|---|
| I | Luc | 8 | 0.756 ± 0.063 | <0.001 |
| | IL-12 | 8 | 0.368 ± 0.036 | |
| II | Luc | 8 | 0.567 ± 0.066 | <0.025 |
| | IL-12 | 8 | 0.381 ± 0.030 | |
| | None | 8 | 0.544 ± 0.056 | <0.025 |
| III | Luc | 8 | 0.593 ± 0.07 | <0.01 |
| | IL-12 | 7 | 0.363 ± 0.025 | |

[a] Balb/c mice were injected i.d. with $10^5$ 4T1 tumor cells. On days 7, 10, 13 and 16 post tumor cell implantation, skin overlying the tumor was transfected with 5 μg of IL-12 cDNA or Luc cDNA, or left untreated (Experiment II).

[b] On day 31 after tumor cell implantation, mice were sacrificed and lung weight was determined. The results of three independent experiments are shown.

Table 2. Enhanced production of IFNγ in tumor-draining lymph nodes following IL-12 gene therapy.

| In Vivo Treatment[a] | IFNγ activity (ng/ml)[b] Mean ± SEM | |
|---|---|---|
| None | 5.2 ± 0.7 | |
| | | N.S.[c] |
| Luc | 35.2 ± 15.0 | |
| | | <0.01 |
| IL-12 | 163.6 ± 21.2 | |

[a] Balb/c mice were injected i.d. in the middle of abdomen with $10^5$ 4T1 tumor cells.

On days 7 and 9 post tumor cell implantation, skin overlying the tumor was transfected with 5 μg of IL-12 cDNA or Luc cDNA, or left untreated.

[b] On day 10 post tumor cell implantation, axillary lymph nodes were removed, and $2.5x10^6$ lymphoid cells were placed in culture without stimulation for 48 hr. IFNγ levels in the supernatants were determined by ELISA. The results are presented for 3-5 mice per group.

[C] N.S. - not significant

# FIGURE LEGENDS

**Figure 1.** Under the conditions used, the TS/A tumor line is immunogenic while 4T1 is not. Immunogenicity of TS/A and 4T1 adenocarcinomas. Balb/c mice were injected i.d. on the right side of abdomen with $10^6$ gamma-irradiated TS/A (A) or 4T1 (B) tumor cells twice at an interval of 3 weeks. One month after the second immunization, vaccinated mice and naive control mice were challenged i.d. on the left side of abdomen with $10^5$ corresponding replicating tumor cells, and tumor growth was followed. Mean tumor diameters $\pm$ SEM are shown for 7-8 mice per group.

**Figure 2.** Antitumor effect of IL-12 gene therapy against established primary mammary tumors. This experiment shows that IL-12 gene therapy was effective against an established primary tumor of the moderately immunogenic TS/A line but not against the non-immunogenic 4T1 line. Mice were injected i.d. with $10^5$ TS/A or 4T1 tumor cells. Gene therapy with IL-12 cDNA or Luc cDNA (control) was performed on days 7, 10, 13 and 16 post tumor cell implantation as indicated by arrows. Numbers (0/8, 4/8) indicate mice with completely regressed tumors. Data are mean tumor diameters $\pm$ SEM for 8 mice per group.

**Figure 3.** IL-12 gene therapy in TS/A tumor-bearing mice results in development of immunological memory as tested by a rejection of secondary tumor challenge. Balb/c mice that rejected TS/A tumors following IL-12 gene therapy were injected intradermally one month later with $1x10^5$ of TS/A cells. As a control, the tumor cells were injected into age-matched naive Balb/c mice. Data are presented as the means $\pm$ SEM of 5 mice per group.

**Figure 4.** Increased induction of CTL activity in mice that rejected TS/A tumors following IL-12 gene therapy. Tumor-specific CTL were generated *in vitro* against TS/A tumor cells. Mean $\pm$ SEM of 4 mice per group. Spleen cells from IL-12 gene-treated mice generated significantly higher levels of CTL activity than spleen cells from naive mice ($p<0.005$). Effector-stimulator ratio 30:1.

**Figure 5.** Reduction of lung metastases of a non-immunogenic tumor line following IL-12 gene therapy. Mice were injected i.d. with $10^5$ 4T1 tumor cells. Gene therapy with IL-12 cDNA (bottom of figure) or Luc cDNA (top) was performed on days 7, 10, 13 and 16 post tumor cell implantation, and the lungs removed on day 31. A photograph of four representative lungs from each group is shown.

**Figure 6.** IL-12 gene therapy increases survival from metastasis of a non-immunogenic tumor. Mice showed significantly increased survival times (see text) after IL-12 gene therapy followed by excision of the primary tumor. Mice were injected i.d. with $10^5$ 4T1 tumor cells. Mice were untreated, or treated with IL-12 cDNA or Luc cDNA on days 3, 7 and 10 (A) or days 4, 7 and 10 (B) post tumor cell implantation, and the primary tumor was excised on day 11. Survival of the mice was followed. Data from two independent experiments are presented for 12 mice per group (Expt. A) and 16 mice per group (Expt. B).

**Figure 7.** IL-12 gene therapy has an anti-metastatic effect against the 4T1 tumor in athymic nude mice. Euthymic Balb/c mice and athymic nude mice were injected i.d. with $10^5$ 4T1 tumor cells. Gene therapy with IL-12 cDNA or Luc cDNA was performed on days 7, 10, 13 and 16 post tumor cell implantation. The lungs were removed on day 31, and their weight was determined. Data are mean lung weight $\pm$ SEM for 8 mice per group.

**Figure 8.** Anti-metastatic effect against 4T1 is retained in T-depleted mice. Balb/c mice were injected i.d. with $1x10^5$ 4T1 cells. Skin was transfected with IL-12 or Luc cDNA expression vectors on days 7, 10,13 and 16 post tumor implantation. Both anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43), each at a dose 300 μg/mouse, were administered intraperitoneally on days 6, 11 and 16 after tumor implantation. Control groups included mice that were treated with the IL-12 gene and received rat IgG (Sigma) at the same doses and schedule as the anti CD8-and CD4 mAb, or mice treated with the Luc gene instead of the IL-12 gene. Mean Lung Weight $\pm$ SEM are shown for 8 mice per group on day 31 post tumor cell implantation.

**Figure 9.** IL-12 gene therapy results in tumor-inhibitory effect in tumor-draining lymph nodes. Mice received skin transfections with IL-12 cDNA or Luc cDNA on days 7 and 10 post 4T1 tumor cell implantation. On day 11, axillary lymph nodes were removed, and TDLN cells pooled from 4 mice per group and cultured in medium without stimulation for 48 hr. Different dilutions of cell-free supernatants were added to a culture of 4T1 cells, and tumor cell proliferation was measured 48 hr later by $^3$H-thymidine uptake. (A) A dose-dependent, tumor-inhibitory activity of TDLN cell supernatants was found after IL-12 gene therapy. Means of Proliferation Inhibition Index (%) of four mice per group. (B) The observed inhibitory activity of TDLN cell supernatants was not neutralized by anti-IFNγ mAb, whereas tumor-inhibitory activity of rMuIFNγ (6 ng/ml) was neutralized. Numbers indicate the Proliferation Inhibition Index (%). Data are means $\pm$ SEM of triplicates.

Figure 1

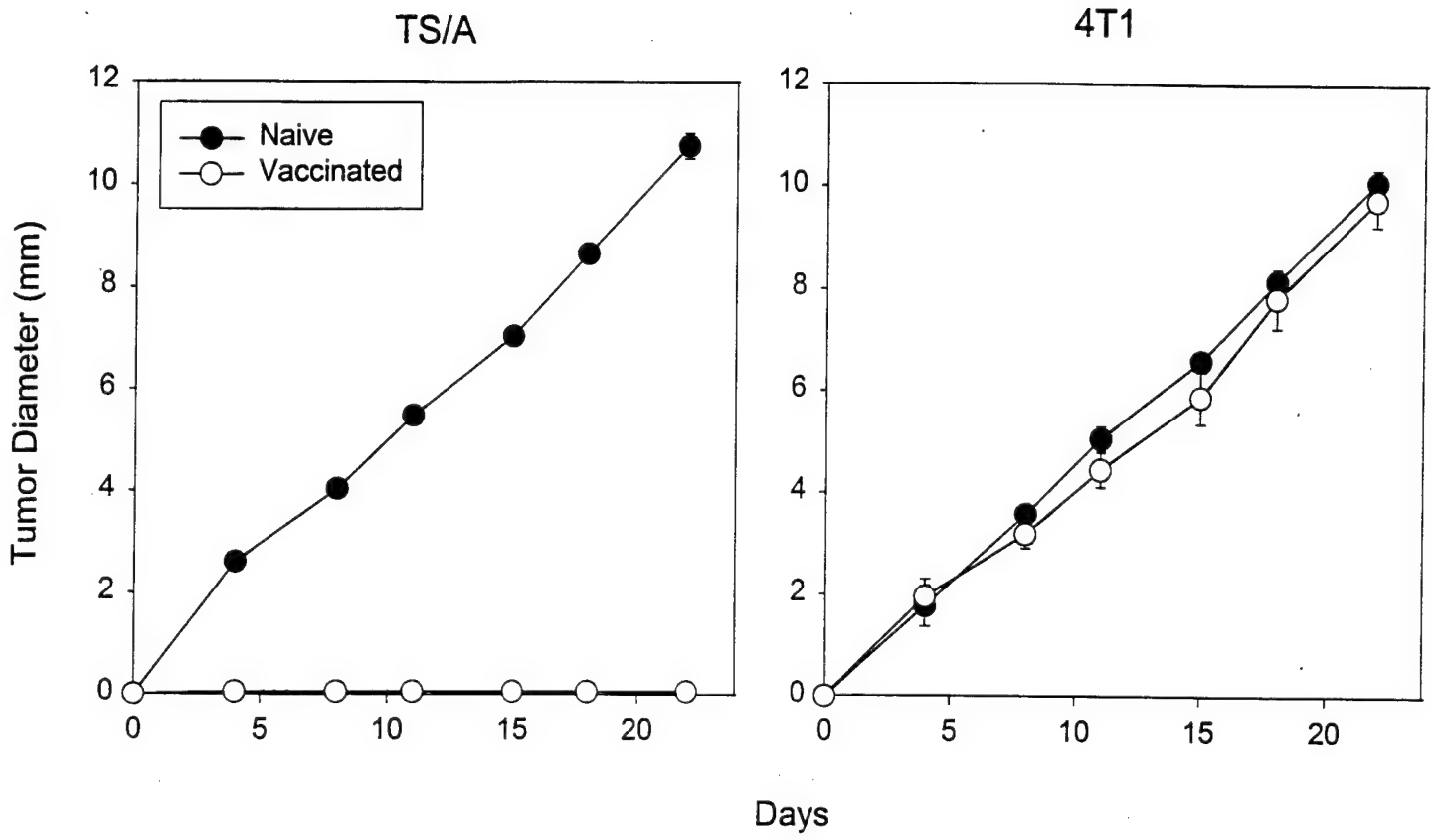

TS/A
4T1
Naive
Vaccinated
Tumor Diameter (mm)
Days
0
2
4
6
8
10
12
0
5
10
15
20

Figure 2

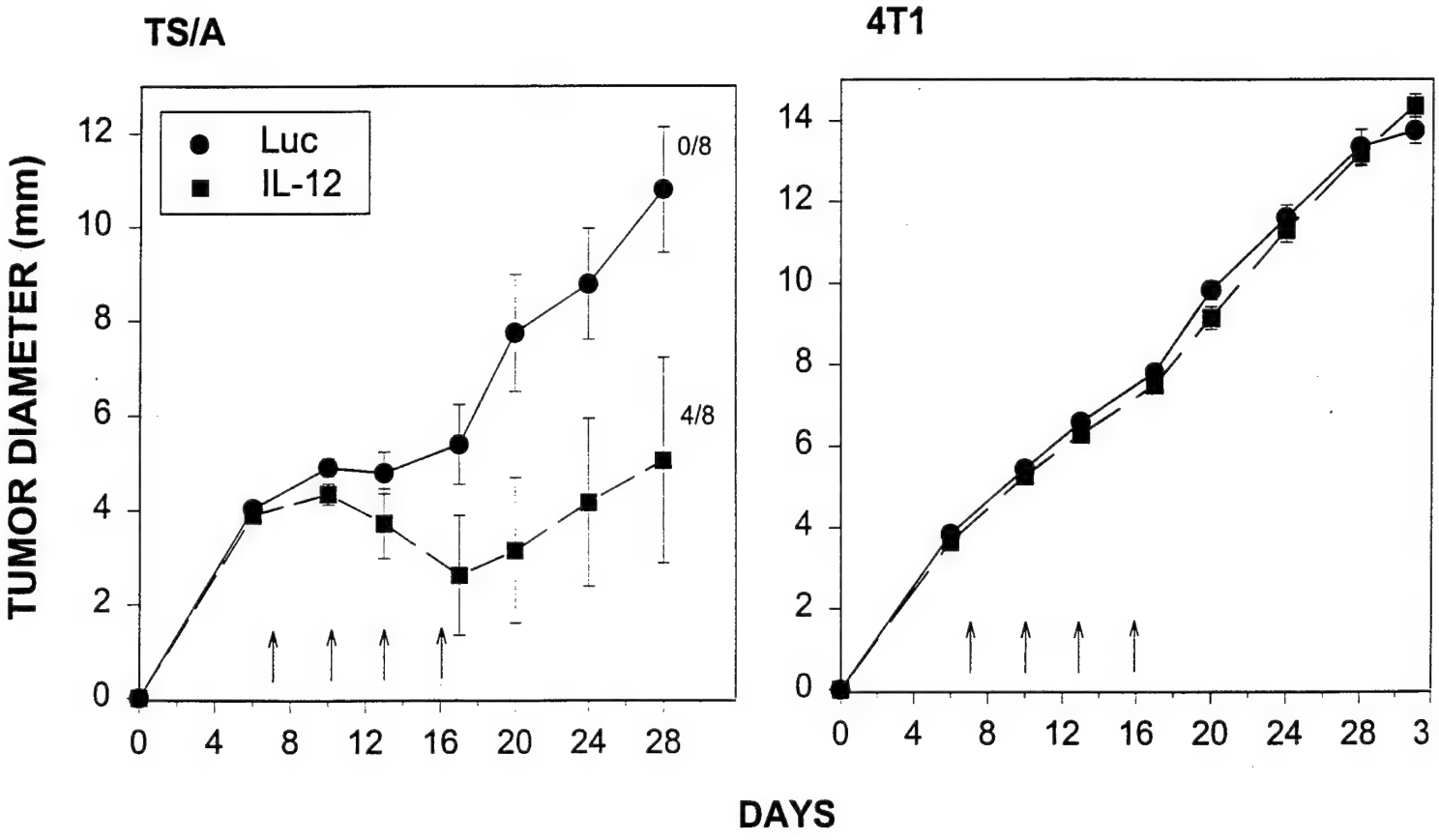

TS/A
4T1
TUMOR DIAMETER (mm)
Luc
IL-12
0/8
4/8
0
2
4
6
8
10
12
14
0
4
8
12
16
20
24
28
DAYS

Figure 3

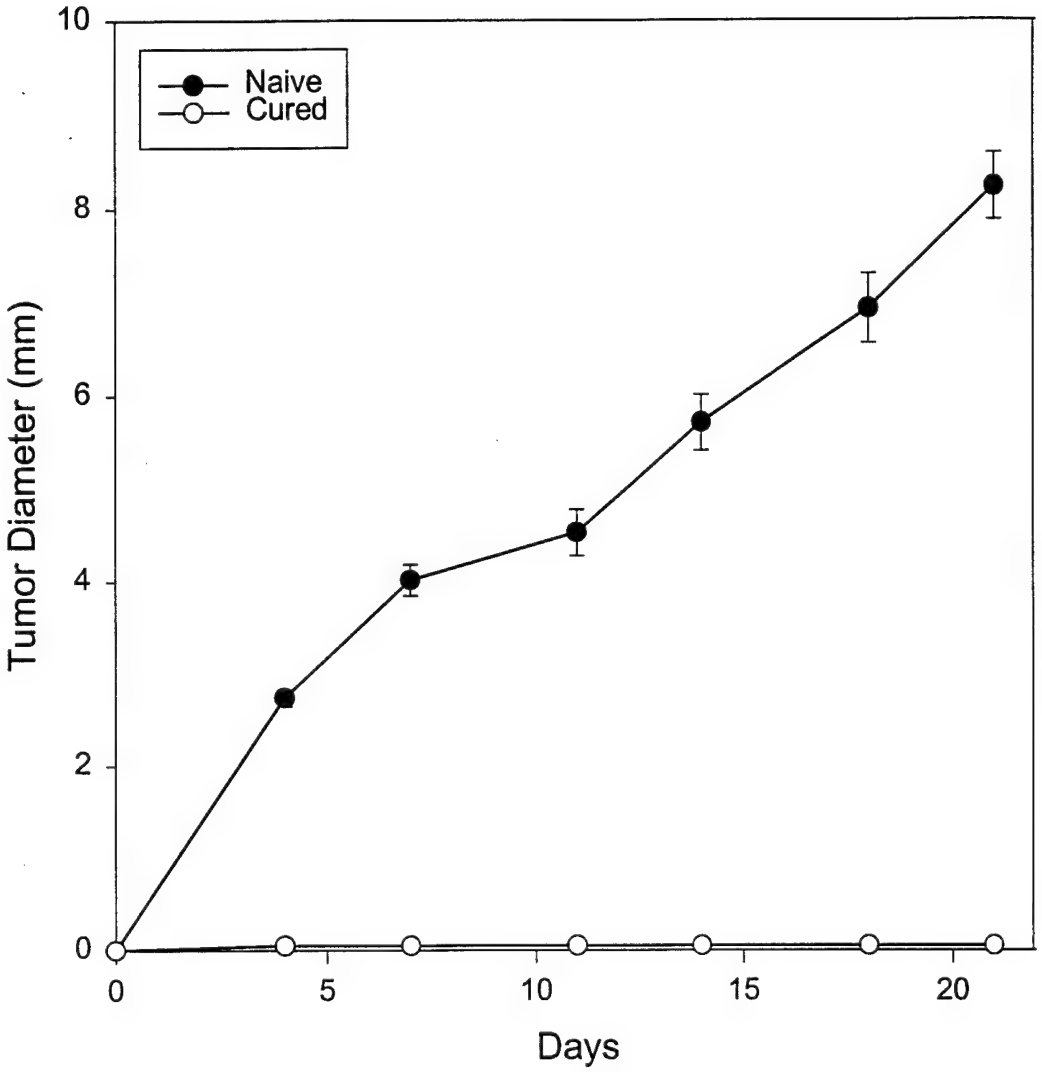


Figure 4

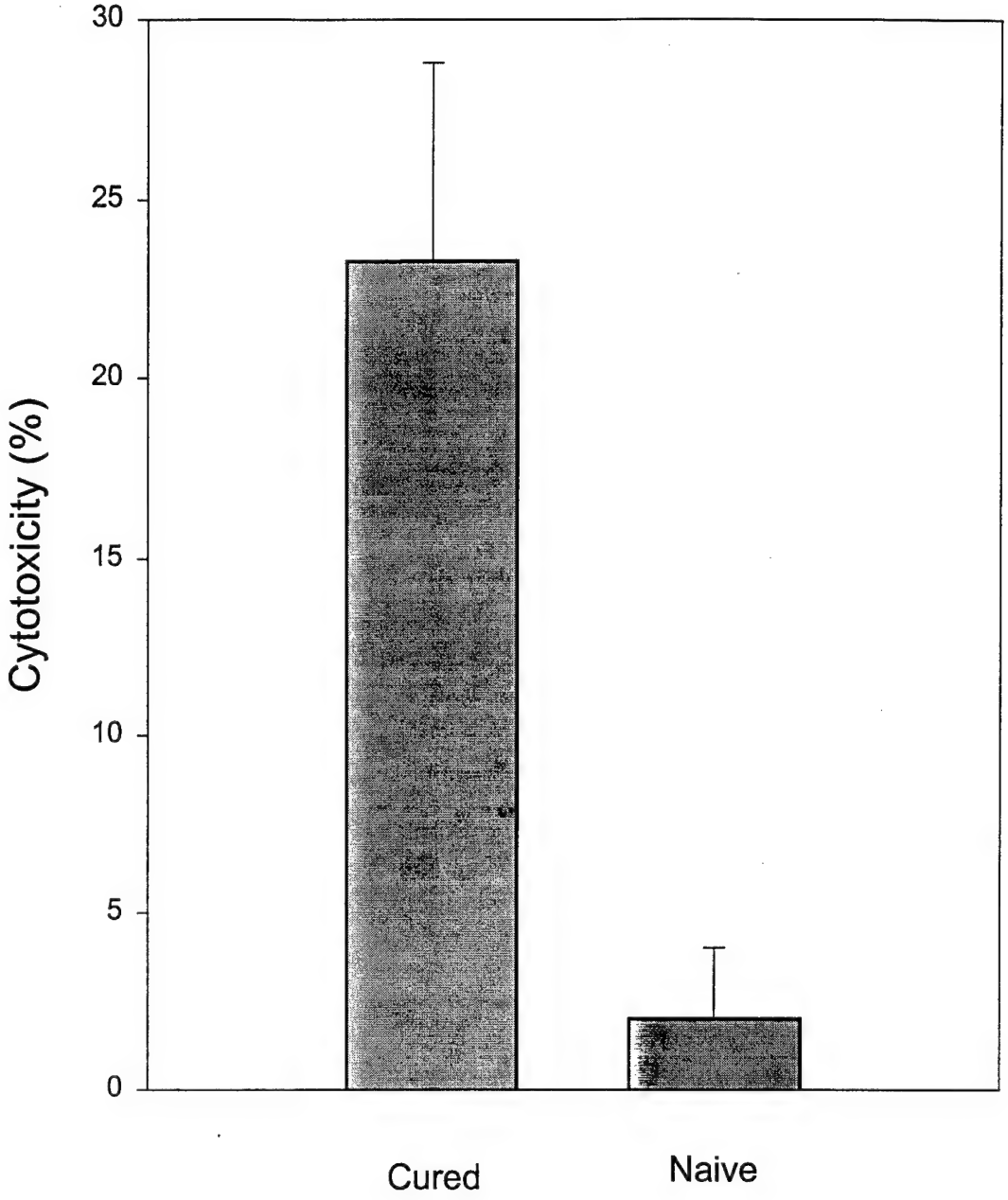


Figure 4

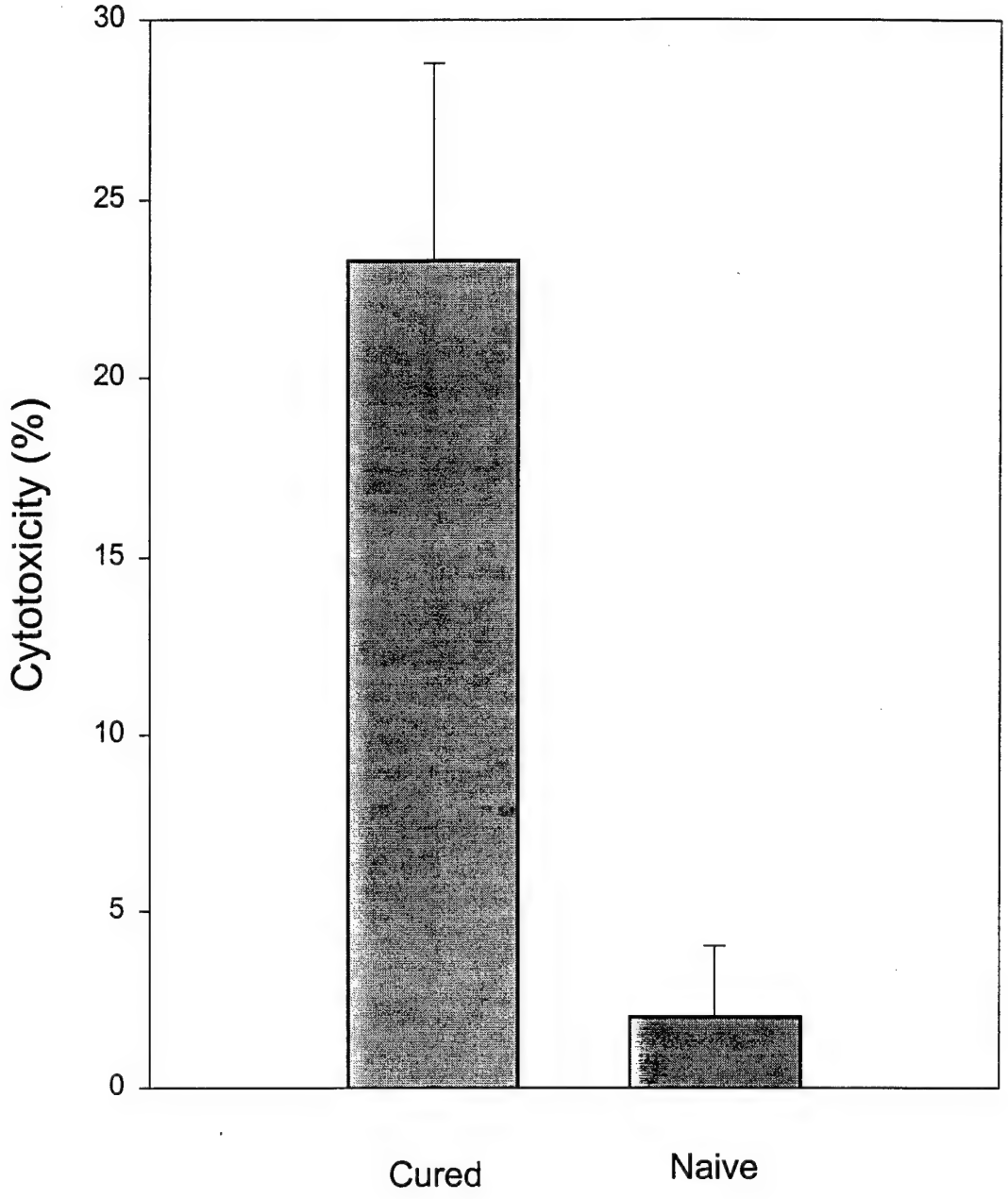


Figure 5

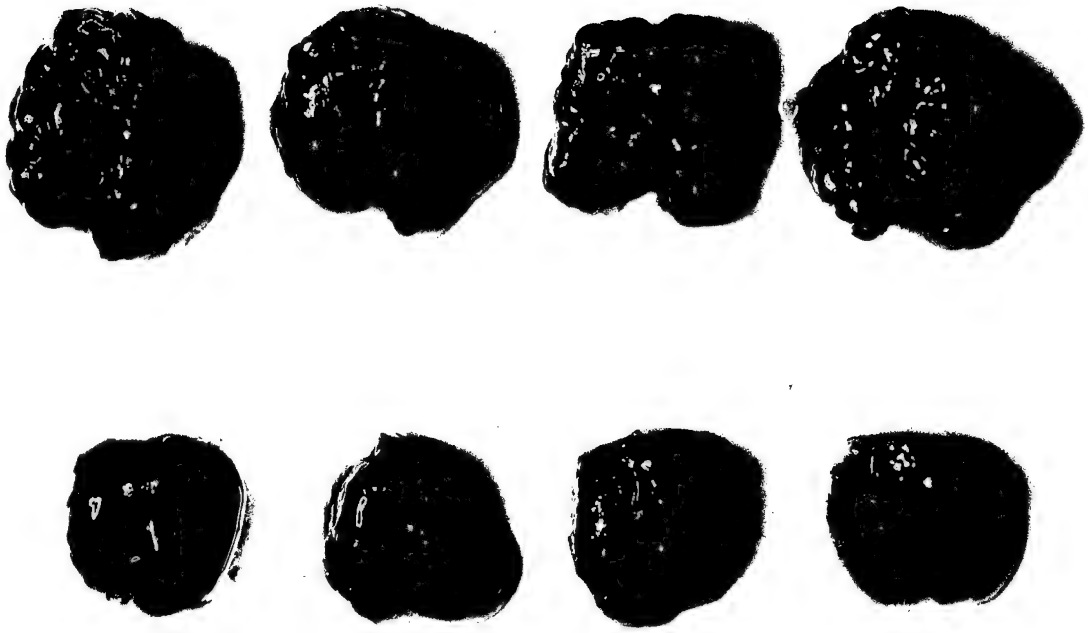
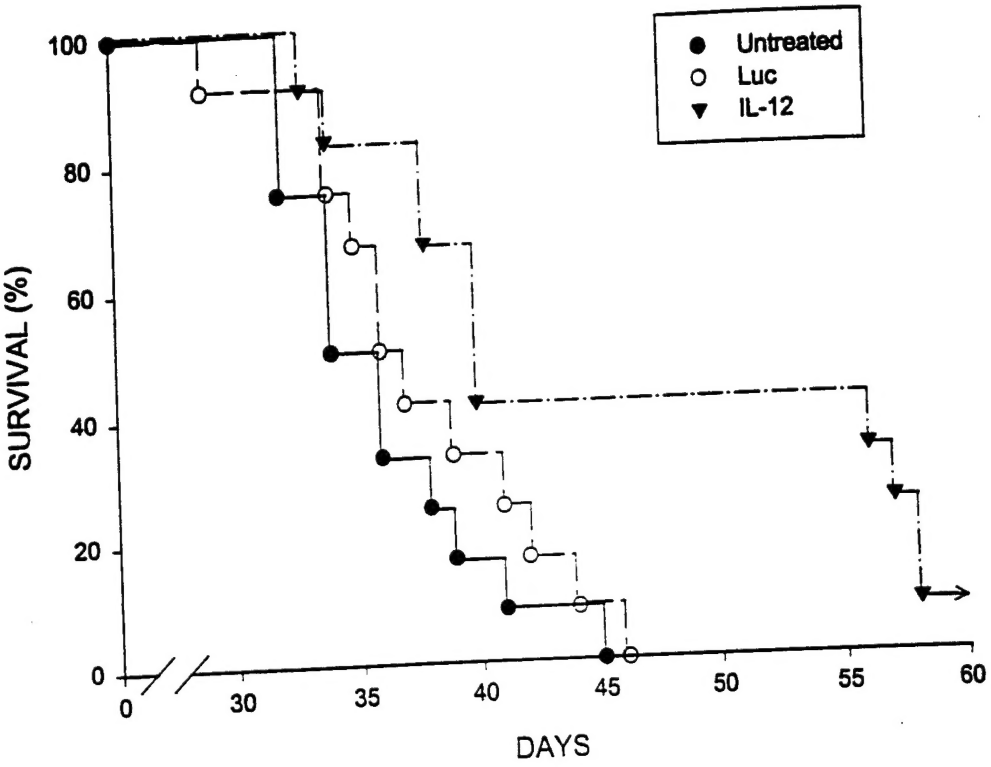


Figure 6

# Experiment 1




# Experiment 2

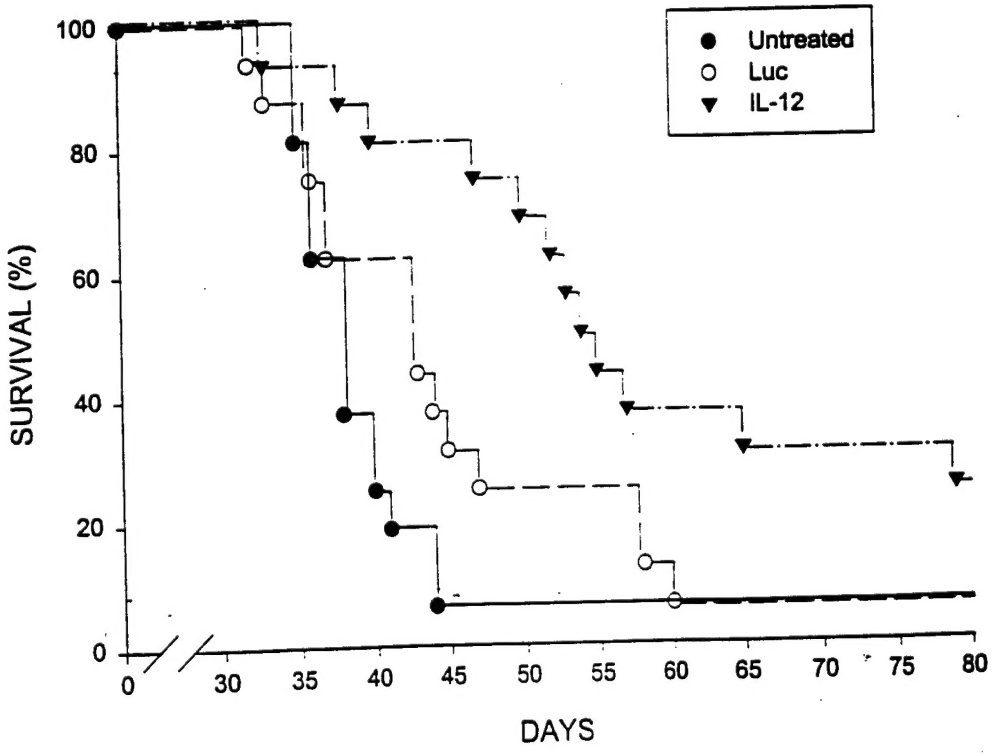


Figure 7

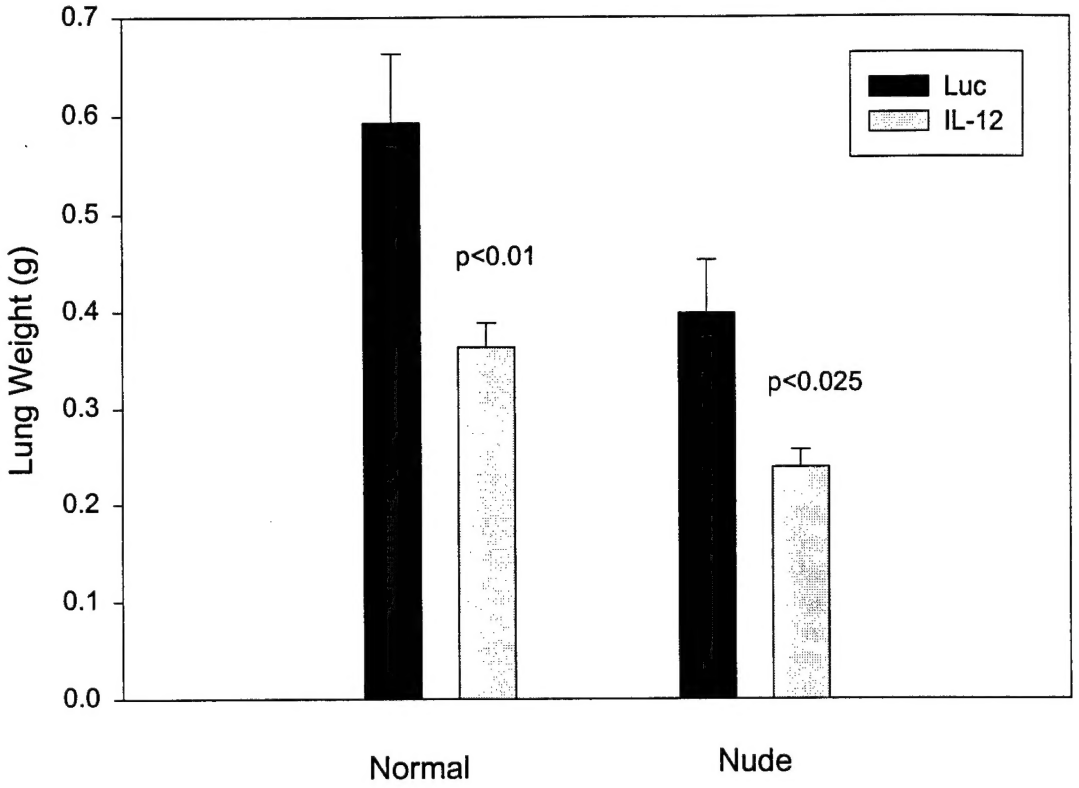

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Lung Weight (g)
Luc
IL-12
p<0.01
p<0.025
Normal
Nude

Figure 8

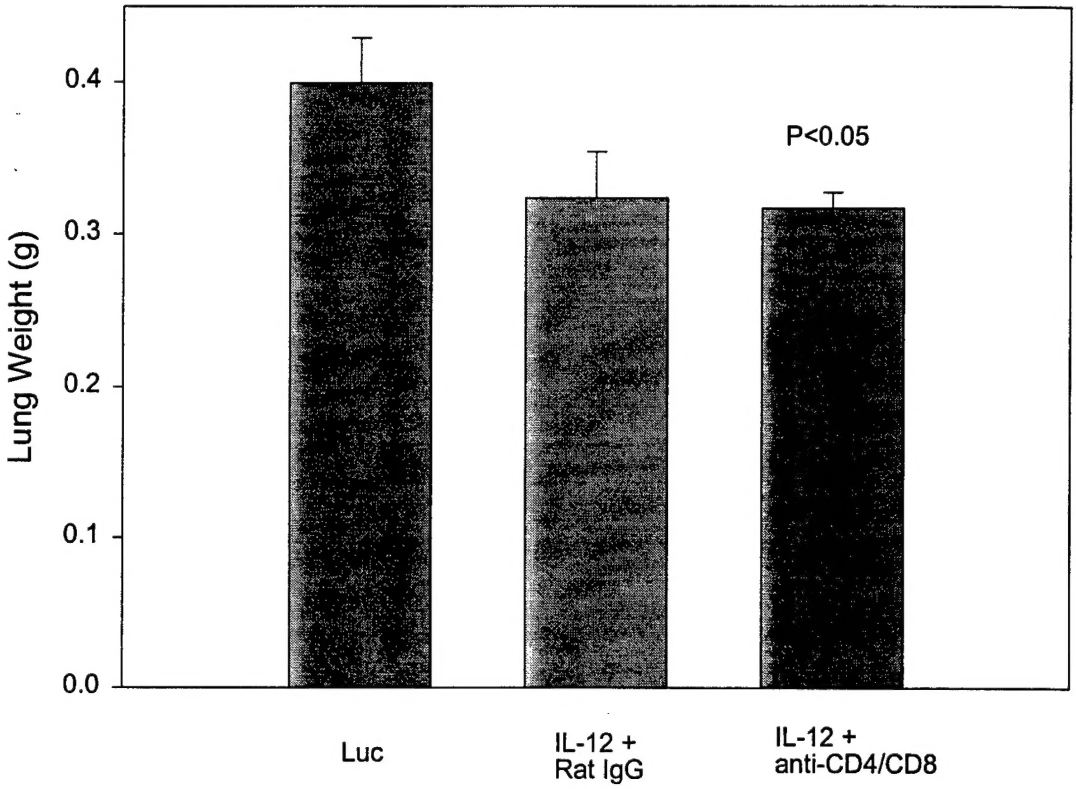
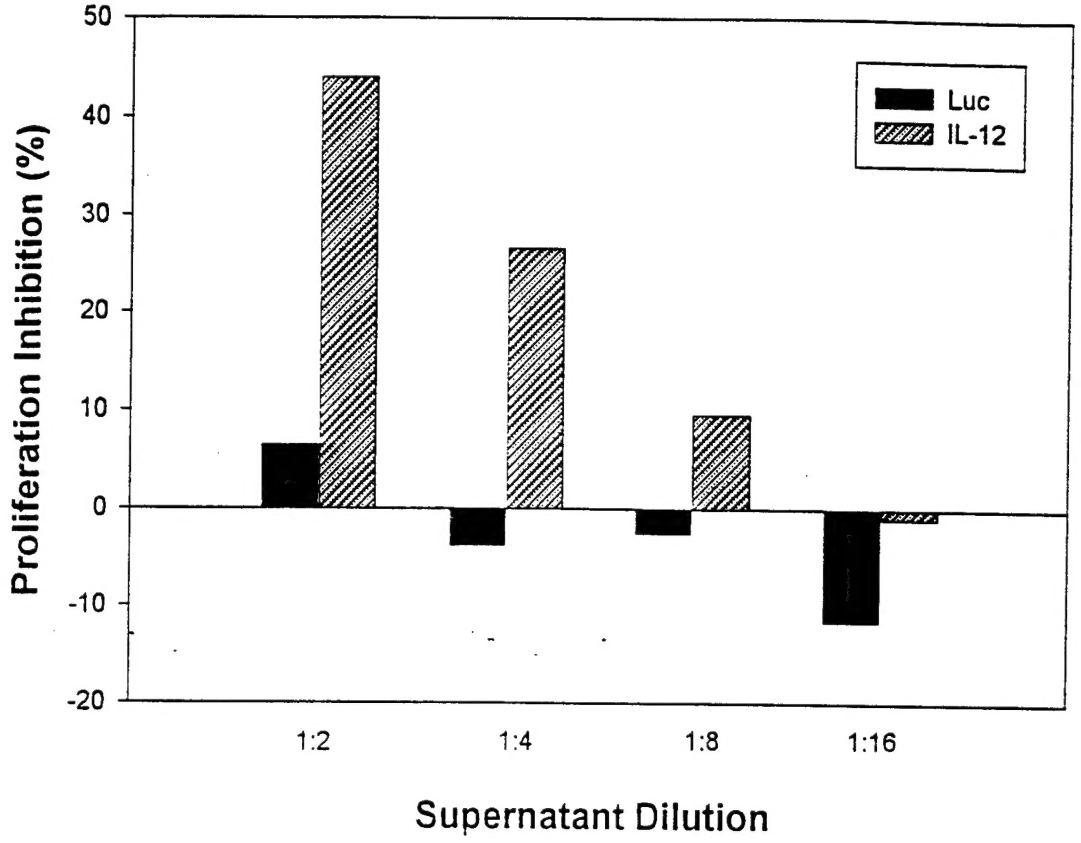
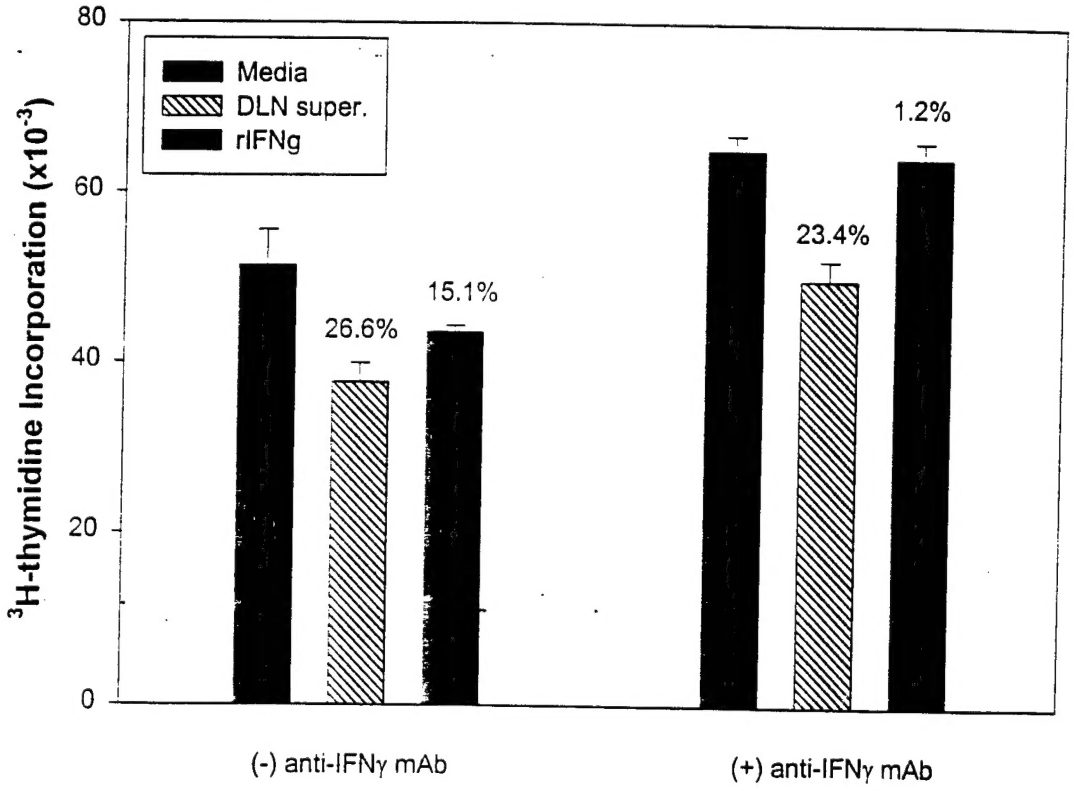

Lung Weight (g)
0.4
0.3
0.2
0.1
0.0
P<0.05
Luc
IL-12 +
Rat IgG
IL-12 +
anti-CD4/CD8

Figure 9

A


50
40
30
20
10
0
-10
-20
Proliferation Inhibition (%)
Luc
IL-12
1:2
1:4
1:8
1:16
Supernatant Dilution

B


80
60
40
20
0
$^3$H-thymidine Incorporation (x10$^{-3}$)
Media
DLN super.
rIFNg
26.6%
15.1%
23.4%
1.2%
(-) anti-IFNγ mAb
(+) anti-IFNγ mAb